The Role of p53 in Suppression of KSHV Cyclin-induced Lymphomagenesis

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ABSTRACT

Kaposi’s sarcoma-associated herpesvirus (KSHV) encodes a cyclin D homolog, K cyclin, that is thought to promote viral oncosensitivity. However, expression of K cyclin in cultured cells not only triggers cell cycle progression but also engages the p53 tumor suppressor pathway, which probably restricts the oncosensitive potential of K cyclin. Therefore, to assess the tumorigenic properties of K cyclin in vivo, we transgenically targeted expression of K cyclin to the B and T lymphocyte compartments via the Eμ promoter/enhancer. Around 17% of Eμ-K cyclin animals develop lymphoma by 9 months of age, and all such lymphomas exhibit loss of p53. A critical role of p53 in suppressing K cyclin-induced lymphomagenesis was confirmed by the greatly accelerated onset of B and T lymphomagenesis in all Eμ-K cyclin/p53−/− mice. However, absence of p53 did not appear to accelerate K cyclin-induced lymphomagenesis by averting apoptosis: Eμ-K cyclin/p53−/− end-stage lymphomas contained abundant apoptotic cells, and transgenic Eμ-K cyclin/p53−/− lymphocytes in vitro were not measurably protected from DNA damage-induced apoptosis compared with Eμ-K cyclin/p53+/+ cells. Notably, whereas aneuploidy was frequently evident in pre-lymphomatous tissues, end-stage Eμ-K cyclin/p53−/− tumors showed a near-diploid DNA content with no aberrant centrosome numbers. Nonetheless, such tumor cells did harbor more restricted genomic alterations, such as single-copy chromosome losses or gains or high-level amplifications. Together, our data support a model in which K cyclin-induced genome instability arises early in the pre-tumorigenic lymphocyte population and that loss of p53 licenses subsequent expansion of tumorigenic clones.

INTRODUCTION

Many DNA tumor viruses encode proteins that engage the host cell cycle machinery and consequently elicit cell cycle progression. For example, the gammaherpesviruses of the Rhadinovirus subfamily, such as the squirrel monkey herpesvirus saimiri (HVS) (1), murine gammaherpesvirus 68 (MHV68) (2), and human Kaposi’s sarcoma-associated herpesvirus (KSHV) (3), all encode their own viral cyclin D homolog. In the case of KSHV, infection of endothelial and B cells is causally linked to two forms of neoplasia, Kaposi’s sarcoma (KS) and various B-cell lymphoproliferative disorders (4–6). In each neoplasm, the great majority of tumor cells are latently infected and express the KSHV cyclin (K cyclin), implicating K cyclin as a key KSHV oncoprotein (7).

The K cyclin (ORF72) sequence shares 53% similarity with cyclin D2 (8) and, similar to D cyclins, directs kinase activity toward Rb when complexed with cyclin-dependent kinases Cdk4 or Cdk6 (9, 10). However, in contrast to cyclin D, K cyclin/Cdk6 complexes exhibit a more promiscuous substrate specificity that includes the S-phase Cdk2 substrates p27Kip1, Cdc6, and Orc1 (11–13). Furthermore, K cyclin/Cdk6 complexes are less susceptible to inhibition by either the Cip/Kip or Ink4 families of CDK inhibitors (14). The K cyclin/Cdk6 complex therefore acts as a constitutively active mimic of the G1 and S-phase cyclin/Cdks.

Consistent with these biochemical properties, addition of K cyclin to isolated nuclei initiates DNA replication (12). Moreover, ectopic expression of K cyclin triggers S-phase entry in quiescent cells or in cells overexpressing p16INK4a or p27Kip1 Cdk inhibitors (14). Nonetheless, cell cycle transition still depends on the endogenous host cell cycle machinery because activation of endogenous Cdks is required for full S-phase progression, even after K cyclin-directed phosphorylation and consequent degradation of p27Kip1 (11, 13). Furthermore, although K cyclin/Cdk6 complexes are resistant to Ink4 proteins (15), they enable S-phase entry only after they have been phosphorylated by cellular Cdk-activating kinase (16, 17). Thus, K cyclin expression promotes cell cycle progression in a manner dependent upon endogenous Cdk activities for full G1-S progression.

The oncogenic potential of K cyclin-induced cell cycle progression appears to be counterbalanced by concomitant activation of growth arrest and apoptotic pathways, the activation of which is clearly evident in K cyclin-expressing cells in vitro (18). K cyclin-dependent apoptosis may, in part, involve phosphorylation and consequent inhibition of Bcl-2 function, which exposes the mitochondrial apoptotic pathway to activation (19). In addition, K cyclin causes p53 accumulation, which is also likely to contribute to both apoptosis and growth arrest (18). Consistent with a key role for p53 in restraining K cyclin-dependent tumorigenesis, we showed recently that K cyclin-expressing fibroblasts exhibit a profound cytokinesis defect, yet continue to initiate and transit S-phase leading to the generation of polyploid cells. Such polyploidy correlated with amplification of centrosomes, the microtubule-organizing centers of the mitotic spindle, and consequent appearance of aneuploidy (18). Such K cyclin-expressing cells were able to divide and survive as an aneuploid population only in the absence of p53, which suggests that the principle role of p53 loss in restraining K cyclin-dependent tumorigenesis is to potentiate the ability of genetically aberrant cells to propagate. We tested this hypothesis in vivo by transgenic expression of K cyclin in mice.

KSHV infection of B lymphocytes predisposes to the development of lymphoproliferative disorders such as primary effusion lymphoma (4) and multicentric Castleman’s disease (6). In addition, the principle Cdk6 binding partner of K cyclin is abundantly expressed in lymphocytes (20), and thymocytes are known to be susceptible to induction of apoptosis by likely K cyclin effectors such as E2F1 and p53 (21–23). For all these reasons, we chose to target K cyclin expression to the lymphocytic compartment of transgenic mice using the well-characterized Eμ enhancer.

In this study, we describe the generation and characterization of Eμ-K cyclin transgenic mice. In keeping with our previous in vitro studies (18), we found that the p53 pathway is obligatorily disrupted in lymphomas arising in Eμ-K cyclin mice. Moreover, Eμ-K cyclin mice lacking p53 exhibited a dramatic decrease in tumor latency and increase in tumor incidence. Nonetheless, inactivation of p53 does not appear to accelerate lymphomagenesis by significantly mitigating apoptosis. Interestingly, although we observed a high proportion of aneuploid or polyploid cells within the pre-tumor Eμ-K-cyclin/p53−/− lymphocyte population, the eventual lymphoma cells all have...
a near diploid chromosome content with no overt aberrations in centrosome number. Closer inspection revealed that such lymphoma cells have frequent and clonal single-copy chromosome gains and losses and high-level amplifications. Our data are consistent with a model in which the pivotal tumor-suppressive role of p53 in restraining K cyclin-induced lymphomas is to curtail outgrowth of genomically aberrant cells during the early stages of lymphomagenesis.

MATERIALS AND METHODS

Cell Culture and Electroporation of DNA. A20 mouse B-cell lymphoma cells were maintained as suspension cultures between 2.5 × 10^6 and 1 × 10^6 cells/ml in RPMI 1640 (Invitrogen) supplemented with 50 μM 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum (FBS). Cells were cultured at 37°C in the presence of 7.5% CO2 and fed daily by addition of fresh RPMI 1640 (1:2). Cells were split every 3 days and plated at a density of 2.5 × 10^6 cells/ml. Before electroporation, cells were resuspended in RPMI 1640 supplemented with 10% FBS (10 × 10^6 cells/500 μl medium/sample). A titrated amount of plasmid DNA was added to the suspension of cells, and the mixture was electroporated at a 310 V, capacitance of 960 μF into the male Thymi, spleens, or lymph nodes were obtained by dissection.

Monitoring K Cyclin-dependent Tumorigenesis. Mice were monitored for 110 to 130 weeks of age for tumor development. Sick mice were euthanized by CO2 asphyxiation, followed by cervical dislocation and/or bilateral thoracotomy.

Isolation, Surface Antigen Staining, and Flow Cytometric Analysis of Lymphocytes. Thymini, spleens, or lymph nodes were obtained by dissection. Tissue was disaggregated by pressing through a 70-μm nylon mesh cell strainer in DMEM containing 2% FBS to obtain a single cell suspension. Spleenic erythrocytes were eliminated by incubation for 5 min at room temperature in ACK buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.4), 150 mM NaCl, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, washed twice and finally resuspended in 400 μl of blocking solution. Surface antigen expression was determined by flow cytometry and analyzed using CELLQuest software.

Immunoprecipitation and Immunoblotting of Cellular Proteins. To immunoprecipitate antigens, primary lymphocytes were lysed with RIPA buffer [1% Triton X-100, 0.5%, sodium deoxycholate, 0.1% SDS, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 0.01 mg/ml aprotinin, and 1 μM EDT], and 250 μg of total protein per sample immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma), followed by collection of immune conjugates on Protein G-Sepharose beads (Sigma). Bound proteins were eluted in Laemmli sample buffer, fractionated by SDS-PAGE, and analyzed by immunoblotting with EV-4 anti-K cyclin antibody (see below).

For direct immunoblotting analysis, protein lysates were prepared by boiling briefly in SDS lysis buffer (2.5% SDS in 0.5 M Tris-HCl, pH 6.8, sonicated, and cleared by centrifugation. Protein concentrations were determined using a Bio-Rad DC assay according to the manufacturer’s protocol. Twenty μg of total protein were fractionated by SDS-PAGE and Western blotting and the filters were probed with polyclonal antibodies directed to mouse p53 (CM5; Novocastra) or p21Cip1 (Pharmingen). Bound antibody was visualized by enhanced chemiluminescence.

Preparation of Anti-K Cyclin Rabbit Polyclonal Antibody. An overnight culture of DH5α bacteria transformed with pGEX-KG K cyclin plasmid was dialyzed in 10°C to fresh Luria broth plus 50 μg/ml ampicillin and grown at 37°C while shaking at 150 rpm until A600 reached 0.6–0.7, and then at 25°C until A600 reached 1.3–1.4. Isopropyl-1-thio-β-D-galactopyranoside (25 μM) was added, and the cultures were incubated overnight at 18°C with constant shaking at 150 rpm. Gluthathione S-transferase fusion proteins were isolated from bacteria and bound to glutathione-Sepharose beads according to the
manufacturer’s instructions (Pharmacia). The glutathione S-transferase portion was removed by overnight incubation of the resin at 16°C in 2 ml of thrombin buffer (1 mM CaCl₂, 50 mM Tris, pH 8) containing 5 units of thrombin enzyme. The purified K cyclin immunogen was used to immunize rabbits using a standard protocol (25). After six rounds of repeated immunization, the animals were exsanguinated, and the final antisera, designated EV-4, was validated by Western blotting against cells expressing K cyclin.

**Immunohistochemical Analysis of Centrosomes.** Lymphocytes isolated from tumors or age- and litter-matched control tissues were washed with PBS/1% BSA. Samples (1 × 10⁶ cells in 200 μl of PBS/1% BSA) were spun onto glass slides by centrifugation for 5 min at 800 rpm using a cytopsin centrifuge. Cells were air dried and fixed by incubation for 6 min in ice-cold methanol at −20°C. Samples were then stained with anti-γ-tubulin antibody (Sigma) as described previously (18).

**Propidium Iodide (PI) Staining and Analysis of Primary Lymphocytes.** To assess the DNA profile of lymphocytes, cells were washed twice with PBS, and cell pellets were fixed in 70% ethanol at 4°C. Fixed cells were resuspended in PBS containing 10 μg/ml PI, 100 units/ml RNase A (Sigma), and 0.1% glucose and analyzed by flow cytometry after 30–60 min.

**Histology Images.** Tissues were fixed by overnight incubation at 4°C in 10% neutral buffered formalin solution (Sigma), rinsed in PBS for 5–10 min, and dehydrated by sequential 45-min washes in 30, 50, and 70% ethanol. Tissues were embedded in paraffin, sectioned (5 μm), and stained with H&E. Histology images were made using a Zeiss Axiosplan 2 imaging microscope and Axiovision software.

**Thymocyte Viability Assay.** Thymocytes from 6- to 10-week-old mice were plated in RPMI 1640 containing 10% heat-inactivated FBS and antibiotics, at a density of 1 × 10⁶ cells/ml. Two to 3 h later, cells were exposed to 3 Gy of γ-irradiation from a Cesium source (Mark 1, Model 68 SN.100; J. Shepherd & Associates). Cells were cultured at 37°C, 5% CO₂, 90% humidity, and cell pellets were fixed in 70% ethanol for 4°C. Fixed cells were resuspended in PBS containing 10 μg/ml PI, 100 units/ml RNase A (Sigma), and 0.1% glucose and analyzed by flow cytometry after 30–60 min.

**RESULTS**

**Generation of Eμ-K Cyclin Transgenic Mice.** To assess the tumorigenic potential of K cyclin and to dissect in vivo processes that may contribute to K cyclin-induced lymphomagenesis, we generated mice in which constitutive K cyclin is targeted to lymphocytes using the well-characterized Eμ enhancer. A cDNA fragment encoding FLAG-tagged K cyclin protein was cloned into pHSE3 plasmid, creating K cyclin pHSE3. This construct drives transgene expression from the chimeric H-2K promoter/IgH enhancer cassette (Fig. 1A; Ref. 18). Expression of FLAG-tagged K cyclin was confirmed in mouse A20 B cells transiently transfected with K cyclin pHSE3 plasmid (Fig. 1B). Linearized, purified pHSE3 K cyclin plasmid was injected into the fertilized oocytes of CBA × C57BL/6 (F₂) founder animals (24). Offspring were screened for integration and transgenic genotype analyses of Eμ-K cyclin transgenic mice. Tail-snip DNA was analyzed by PCR or Southern blotting to identify K cyclin DNA. +, positive control PCR on KpcDNA3 plasmid. C, expression of FLAG-tagged K cyclin from the transgenic construct in A20 mouse B lymphocytes electroporated with various amounts of plasmid. KpcDNA3 was used as a positive control. Protein lysates were resolved by SDS-PAGE and immunoblotted using anti-FLAG antibody. D, expression of K cyclin protein in Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) and used to identify K cyclin antibody. E, Western blot. Eμ-K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot. F, expression of K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot. G, expression of K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot. H, expression of K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot. I, expression of K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot. J, expression of K cyclin protein in splenic B and T lymphocytes from Eμ-K cyclin 1996E line 6 mice. Lymphocytes were prepared from spleens or thymi of mice 8–10 weeks of age. Proteins were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted using anti-K cyclin antibody. Western blot.
types. Eμ-K cyclin line 6 spleens were disaggregated, and T cells were identified with antibody specific for receptor β chain [T-cell receptor β (TCRβ)], whereas B cells were identified using antibody specific for the pan-B cell marker CD45R/B220. Different lymphocyte populations were then separated by fluorescence-activated cell sorting (FACS) and assayed by immunoblotting. Both TCRβ-positive and B220-positive splenic cell populations expressed K cyclin protein (Fig. 1E), demonstrating that all peripheral lymphocytes express K cyclin in this mouse line.

**Constitutive Expression of K Cyclin in Lymphocyte Expression Is Lymphomagenic.** Transgenic Eμ-K cyclin and control mice were monitored for the development of lymphomas during their first year of life. None of the control mice developed lymphomas, in agreement with the established refractoriness of C57BL/6 mice to lymphoma induction. The lymphoid compartment promotes the development of both B- and T-cell lymphomas.

The relatively low incidence and late onset of lymphoma in Eμ-K cyclin mice suggested that additional sporadic somatic mutations are required for lymphomas to arise. Because K cyclin expression in vitro triggers a p53-dependent growth arrest (18), inactivation of the p53 gene is a strong candidate cooperating lesion. We therefore examined whether p53 levels are up-regulated in K cyclin lymphomas. Functionally inactive mutant forms of p53 accumulate to supraphysiologic levels because of failure to induce expression of the Mdm2 gene, which encodes the ubiquitin ligase responsible for p53 degradation (32, 33). Immunoblotting analysis demonstrated high levels of p53 in four of five of the analyzed tumors (Fig. 2D). Furthermore, cells from all five tumors exhibited elevated levels of p19ARF protein, an inhibitor of Mdm2-mediated ubiquitination and degradation of p53 (34). This is again consistent with p53 being functionally inactive, because cells containing wild-type p53 contain undetectable levels of p19ARF, explained by a p53-mediated negative feedback loop (35). Importantly, the increases observed in p53 and p19ARF were not a consequence of K cyclin expression per se, because their levels remained low in Eμ-K cyclin transgenic splenocytes and thymocytes in prelymphomatous mice (Fig. 2D). These data demonstrate that the p53 pathway is disrupted in Eμ-K cyclin lymphomas.

**Eμ-K Cyclin Cooperates with p53 Loss in Lymphomagenesis.** To validate directly the role of p53 loss in promotion of Eμ-K cyclin-induced lymphomagenesis, Eμ-K cyclin transgenic mice were bred into p53−/− heterozygous and p53−/− homozygous backgrounds, and lymphoma incidence was then monitored. Around 17% (3 of 18) control Eμ-K cyclin/p53+/+ transgenic mice developed lymphoma around 7 months of age (Table 1), essentially the same as the incidence recorded in Table 1. Absence of one p53 allele increased the incidence of lymphomas to 50% (16 of 33), with only a slight acceleration in tumor onset averaging, again, 7 months of age. All tested Eμ-K cyclin/p53+/− lymphomas exhibited the second p53 allele (Fig. 3B), illustrating the potency of p53 as a suppressor of K cyclin-dependent tumorigenicity. Nontransgenic p53−/− mice succumbed to a variety of tumors (the majority of which were B- or T-cell lymphomas) around 6 months of age, in agreement with previous reports on tumor development in p53−/− mice (36, 37). However, Eμ-K cyclin/p53−/− mice all rapidly developed lymphomas with a median latency of only 2.5–3 months (Fig. 3A).

Most Eμ-K cyclin/p53−/− mice developed aggressive thymic T cell lymphomas (Table 2) in which the thymus had expanded to fill the entire thoracic cavity. Some mice developed B-cell lymphoma, evidence of pronounced splenomegaly comprising cells positive for the B220 pan B-cell marker. B- and T-lymphoma cells had usually disseminated to the lymph nodes and/or liver, and in some instances, T-cell infiltrates were observed in the spleen and B-cell infiltrates in the thymus. Further characterization of the tumors using diagnostic cell surface markers and flow cytometry (Fig. 3, C and D; Table 2).

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**Table 1** Tumor development in Eμ-K cyclin transgenic mice
*Mice were checked for the development of lymphoma until they were 1 year of age.

<table>
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<td>F</td>
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<tr>
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<td>181</td>
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<tr>
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<td>8</td>
<td>F</td>
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* ND, not determined.

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Fig. 2. K cyclin expression predisposes to the development of lymphomas. A, expression of K cyclin protein in tumor cells. Lysates from 10 × 10⁶ thymocytes (T), splenocytes (S), or lymph node cells (L.N.) from Eμ-K cyclin lymphoma 4 and cells from a negative nontransgenic littermate control (n.l.) were immunoblotted with anti-FLAG antibody. +, positive control lysate of Rat-1 cells expressing K cyclin. The lower molecular weight band in normal L.N. and S reflects mouse IgG light chain. B, Eμ-K cyclin lymphoma 4 cells and control thymocytes were analyzed by flow cytometry. Dot plots depict increased forward and side scatter of lymphocytes, which shows that lymphomas consist of enlarged cells. C, cells from Eμ-K cyclin lymphoma 4 and control lymphocytes were stained with anti-CD4 and anti-CD8-PE and anti-TCRβ-FITC and analyzed by flow cytometry. A, K cyclin lymphoma (upper panel) but not in lymphocytes from healthy Eμ-K cyclin transgenic mice (upper panel). Lymphocytes were isolated from healthy transgenics (e.g., tumor cells, human lymphocytes from control negative littermates (n.l.). Protein lysates from these cells were resolved on SDS-PAGE gels (20 μg of protein/lane) and immunoblotted with anti-p53 or anti-p19ARF antibody. A lysate of p53−/− mouse embryonic fibroblasts was loaded as negative control for p53 and positive control for p19ARF protein, because they contain high levels of p19ARF due to the lack of a p53-mediated negative feedback loop (35). T, thymocytes; S, splenocytes.
Fig. 3. K cyclin expression cooperates with p53 loss in lymphomagenesis. A, Eµ-K cyclin transgenic mice and p53−/− mice were crossed to generate K cyclin transgenic mice with p53−/−, p53+/−, or p53+/+ backgrounds. Kaplan-Meier survival curves are shown of mice through 12 months of age [an extension of our study published previously (18)]. B, loss of heterozygosity analysis of Eµ-K cyclin/p53−/− lymphoma DNA. Genomic DNA was isolated from control p53−/− thymocytes (Lane 1) or lymphoma cells, and PCR was performed according to tail-snip genotyping protocols. C, cells isolated from a nontransgenic mouse, 10 weeks of age, were stained with anti-CD4-PE and anti-B220-PE and anti-TCRβ-FITC, with anti-CD8-FITC, with anti-B220-PE and anti-TCRβ-FITC or with anti-CD4-PE and anti-CD8-FITC, and then analyzed by flow cytometry. D, example of the phenotypic analysis of tumor cells. Thymocytes, splenocytes, and inguinal lymph node cells from a p53−/− mouse with a thymic lymphoma were stained and analyzed as described in C. This mouse contained both a T-cell lymphoma (note low levels of TCRβ expression) and a B-cell lymphoma (note that cells were surface IgM negative). The B-cell lymphoma had infiltrated the lymph nodes.

showed that all B-cell tumors lacked expression of surface IgM, a marker of B-cell maturation, whereas most T-lymphoma cells were DP/TCRlow, characteristic of immature (precursor) T cells (38). Histopathological analysis showed that the lymphomas consisted of uniform populations of medium-sized B or T cells with high nuclear to cytoplasmic ratios that completely displaced the normal thymic or splenic structure (Fig. 4). In addition, the thymic lymphomas displayed a “starry sky” pattern of macrophages, probably reflecting increased apoptosis and consequent phagocytosis of dead cells. Indeed, pyknotic cells were visible throughout the thymus, although nearly all were located within enlarged macrophages. We could detect numerous mitotic figures (Fig. 4B), indicative of a high proliferation rate. In the B-cell lymphomas, tumor cells were often found in both circulating blood and lymph nodes, as well as in perportal vein or parenchymal infiltrates of the liver (Fig. 4C), and in some cases, also kidney and lung. These pathological features are typical of precursor lymphoblastic lymphoma (38). Thus, K cyclin expression induces precursor B- or T-cell lymphoblastic lymphoma in p53-deficient mice.

**p53 Inactivation Does Not Measurably Suppress Apoptosis of K Cyclin-Expressing Lymphocytes.** p53-mediated suppression of K cyclin-induced tumorigenesis could plausibly be attributable to induction of apoptosis. To assess the degree of protection from apoptosis afforded by p53 loss in transgenic K cyclin lymphocytes, we assayed the extent of their apoptosis after exposure to γ-irradiation. In agreement with published data (39, 40), p53−/− thymocytes displayed marked radioresistance (Fig. 5). Surprisingly, however, Eµ-K cyclin/ p53−/− thymocytes remained just as sensitive as p53+/− and Eµ-K cyclin transgenic thymocytes to radiation-induced apoptosis. This, together with the very high numbers of apoptotic cells observed in all Eµ-K cyclin/p53−/− thymic lymphomas (Fig. 5B), suggests that global suppression of apoptosis is not the significant mechanism by which p53 inactivation accelerates K cyclin-induced lymphomagenesis.

**Eµ-K Cyclin Tumors Are Pseudo-Diploid Yet Exhibit Genomic Alterations.** In cultured fibroblasts in vitro, expression of K cyclin induces marked and rapid centrosome amplification and polyploidy (18). Whether genome instability is a prerequisite for tumor progression remains a subject of debate. However, it is likely to contribute to tumor incidence and progression whenever it occurs. Inactivation of p53 is widely thought to foster genome instability through loss of critical p53-dependent checkpoints triggered by aberrant chromosome segregation, number, and/or breakage. Thus, although p53 inactivation does not appear to confer appreciable general protection from apoptosis in transgenic Eµ-K cyclin lymphocytes, it could nonetheless cooperate with K cyclin by fostering the outgrowth of genetically aberrant clones early in lymphomagenesis, perhaps by suppressing their apoptosis or permitting their sustained proliferation.

We therefore investigated the status of genome integrity in our Eµ-K cyclin lymphoma cells. Of nine lymphomas arising in nontransgenic p53−/− animals, three exhibited a clear aneuploid component, as determined by PI staining and flow cytometry. However, only 1 of 11 Eµ-K cyclin/p53−/− lymphomas contained an obviously aberrant DNA content, which appeared polyploid rather than aneuploid (Fig. 6A). In addition, we used γ-tubulin staining to mark centrosomes in Eµ-K cyclin/p53−/− lymphomas, and this revealed that ~98% of tumor cells contained a normal complement of one or two centrosomes, a proportion similar to control nontransgenic lymphocytes (Fig. 6B). Thus, we found no evidence of large-scale changes in chromosome copy number in end-stage Eµ-K cyclin tumors.

Nonetheless, it remains possible that centrosome amplification and/or aneuploidy arise early in Eµ-K cyclin/p53−/− lymphoma progression, thereby generating a pool of aberrant lymphomatous progenitors, but that cells with grossly aberrant genomes fail to thrive or are outcompeted by their more “normal” rivals. To explore this possibility, we investigated the status of genome integrity in normal thymocytes from pre-lymphomatous Eµ-K cyclin/p53−/− young animals. Neither p53−/− nor Eµ-K cyclin/p53−/− thymocytes exhibited abnormal numbers of centrosomes (Fig. 6C). However, whereas p53−/− thymocytes all had a near-diploid DNA complement (four

<table>
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<td>4 B-cell lymphoma (16%)</td>
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* ND, not determined. |
samples), four of six Eμ-K cyclin/p53−/− mice contained thymocytes that were clearly aneuploid (Fig. 6D). Furthermore, the remaining two apparently diploid samples exhibited a significant increase in the proportion of cells with S/G2/M DNA content, and their thymi were enlarged and resembled those of mice with thymomas. Taken together, these observations indicate that aneuploidy is relatively frequent in pre-lymphomatous Eμ-K cyclin/p53−/− thymocytes, raising the possibility that tumors arise through clonal expansion of advantageous lesions within the aneuploid population. Consistent with this idea, array CGH of tumor versus reference DNA revealed that all Eμ-K cyclin/p53−/− lymphomas harbored single-copy chromosome gains or losses and, occasionally, high-level amplifications or deletions (Fig. 6E). We therefore propose that the most likely reason why p53 loss is an obligate lesion in Eμ-K cyclin lymphomagenesis is that p53 loss allows the expansion of a genetically altered and proliferative competent subset of Eμ-K cyclin lymphocytes.

DISCUSSION

Many factors implicate K cyclin as a critical oncogenic determinant of KSHV-associated malignancies. However, our previous in vitro studies suggested that such oncogenic potential can only be manifest in the absence of a functional p53 pathway (18) because although K cyclin expression triggers cell proliferation and aberrant ploidy, it also triggers a profound growth arrest and apoptosis in p53-competent cells. Here, we describe the generation of an Eμ-K cyclin transgenic mouse model with which to delineate the interplay between the p53 tumor suppressor and K cyclin in lymphomagenesis in vivo. Our current study confirms that p53 compromises K cyclin-induced tumorigenesis and suggests that loss of p53 appears to allow escape of a subset of a genetically altered Eμ-K cyclin cells that then evolve into lymphomas.

Our most striking conclusion is that Eμ-K cyclin-dependent lymphomagenesis strictly requires inactivation of the p53 pathway. Recently, detailed analysis of Eμ-myc-induced lymphomas has demonstrated that inactivation of the ARF-Mdm2-p53 pathway is essentially a prerequisite for lymphoma development (41). By analogy, we predicted that the p53 pathway would also be disrupted in Eμ-K cyclin lymphomas. Consistent with this notion, constitutive expression of K cyclin in the lymphoid compartment induces lymphomas only infrequently in p53−/− mice, and all of the lymphomas that eventually do arise exhibit highly elevated levels of p53 and/or p19ARF proteins,
indicative of disruption of the Mdm2-p53 and p19ARF-p53 negative feedback loops that operate when the p53 pathway is functional (32, 33, 42). Furthermore, lymphomas arising in Eμ-K cyclin/p53−/− animals all exhibit loss of heterozygosity of the remaining p53 allele. Finally, crossing animals onto a p53 null background substantially decreases the latency and increases the incidence of K cyclin-induced lymphomas.

In addition to Eμ-K cyclin, several other lymphocyte-specific transgenic cyclin models have been reported. Expression of Eμ-directed expression of cyclin D1, unlike K cyclin, does not promote lymphomagenesis on its own, although it significantly accelerates Myc-dependent B- and T-cell lymphomagenesis (28, 43). This divergence in oncogenic potential presumably reflects the profound differences in expression, substrate specificity, and susceptibility to regulation of D1 versus K cyclins. Transgenic targeting of the gammaherpesvirus MHV68-encoded cyclin D homolog (M cyclin) to T cells using the proximal lck promoter induces T-cell lymphomas in ~40% of mice between 3 and 12 months of age (44). It is unknown if the p53 pathway is also disrupted in cellular cyclin D or M cyclin-dependent lymphomas, although requirement for some form of secondary sporadic mutation is consistent with the long latency of M cyclin-induced tumor onset. A caveat of such transgenic studies is that cyclins are overexpressed at potential supraphysiological levels, possibly explaining part of the divergence in results. Furthermore, formation of lymphomas in primates by the related HVS virus in primates was shown to be independent of the HVS-encoded viral cyclin (45), warranting studies addressing K cyclin-induced lymphomagenesis in a viral context.

All K cyclin-associated B-cell and most T-cell lymphomas comprise immature proliferating cells, characterized by their lack of surface IgM or low TCRβ expression, respectively. These markers, together with various pathological attributes, define these neoplasms as precursor lymphoblastic lymphomas. Typically, such immature IgM− B or DP/TCRlow T lymphoma cells are able to escape the boundaries of their normal somatic environment (bone marrow or thymus, respectively) and spread to blood and peripheral organs. Similar attributes are shared by lymphomas arising in many other transgenic models in which oncogenes are targeted to lymphoid tissues, including Eμ-myc (46, 47), Eμ-ret (48, 49), and lck-M cyclin (44). In all cases, it remains unclear whether the primary action of the oncogenic lesion is to promote cell proliferation and/or to suppress differentiation. In many instances, these two biological outcomes are probably inextricably intertwined, as suggested by the fact that inhibition of lymphocyte differentiation, as occurs in mice lacking E2A helix-loop-helix proteins that mediate early thymocyte development, is alone sufficient to predispose to thymic lymphoma (50). Further studies will be needed to dissect out the primary biological target of K cyclin activation in lymphocytes.
p53 has well-described roles in the induction of both growth arrest and apoptosis in response to insult, DNA damage, or activation of oncogenes such as K cycin. Indeed, we showed previously that both K cycin-induced growth arrest and apoptosis in mouse embryonic fibroblasts are p53 dependent (18). Surprisingly, analysis of transgenic lymphocytes from Eμ-K cycin/p53−/− mice indicated no discernible increase in apoptosis compared with p53+/− lymphocytes. In part this probably reflects inherent differences between fibroblasts and lymphocytes and in their requirements for survival factors, although it is also possible that adaptation to constitutive K cycin expression may have occurred during lymphocyte ontogeny. Inactivation of p53 did not measurably modify any aspect of Eμ-K cycin transgenic lymphocyte behavior or disposition. In particular, Eμ-K cycin/ p53−/− lymphocytes retained p53+/− sensitivity to induction of apoptosis. Indeed, even the Eμ-K cycin/p53−/− lymphomas contain significant numbers of apoptotic cells. Thus, loss of p53 appears to have little effect on the propensity for Eμ-K cycin transgenic lymphocytes to undergo cell death.

Expression of K cycin in cultured primary fibroblasts induces centrosome amplification and pronounced aneuploidy, although such cells can propagate only if they lack functional p53 (18). Our studies suggest that K cycin also promotes genomic instability in lymphocytes in vivo: aneuploidy is frequent in pre-tumorigenic Eμ-K cycin/ p53−/− thymocytes compared with nontransgenic p53+/− cells. Surprisingly, however, we observed no significant centrosome amplification in either pre-lymphomatous lymphocytes, in contrast with a previous study (51), or in end-stage Eμ-K cycin/p53−/− lymphomas. Nonetheless, array CGH analysis of end-stage Eμ-K cycin/p53−/− lymphomas revealed frequent whole-chromosome gains and losses, genomic aberrations that are the typical consequences of aberrant centrosome maintenance. Our best guess is that both centrosome amplification and aneuploidy arise transiently during early stages of tumor onset and provide the engine for genomic aberration. However, only those cells with relatively normal chromosomal complement can eventually expand to form tumors, perhaps because successful cell division requires efficient mitosis that favors bipolar spindles (52). Consistent with this, the presence of supernumerary centrosomes has been associated with early-stage neoplastic progression, several cancers including HPV-associated genital lesions (53), and pancreatic cancer in SV40 large T transgenic mice (53, 54). A more fundamental question, not confined to K cycin-dependent tumorigenesis, is whether genomic alterations contribute causally to tumor progression or whether they are merely consequences of inherent checkpoint failure (55). Indeed, it has been suggested recently that the acquisition of aneuploidy does not constitute a critical tumorigenic determinant of p53 loss in the Eμ-myc lymphoma model (56, 57). However, although cells derived from Kaposi sarcoma or primary effusion lymphoma seldom display overt ploidy changes (see examples in Refs. 58–60), it is less clear whether they harbor more restricted genomic abnormalities. Moreover, both KS and primary effusion lymphoma are thought to begin as polyclonal hyperplasias from which monoclonal tumors eventually evolve (61, 62). Our data suggest a model in which sustained expression of K cycin in early hyperplasias provides the engine of genetic diversity from which clonal neoplasms eventually emerge once p53 function has been lost or compromised by other virally expressed proteins. In this context, it is interesting to note that two latent proteins that are tightly coexpressed with K cycin, LANA and v-FLIP, respectively compromise activation of p53 (63) and promote tumorigenesis by inhibiting lymphocyte apoptosis (64). In addition, KSHV-associated disorders do not display recurrent p53 mutations (65–67), and LANA was found to colocalize with p53 in KS samples (66). Together with our findings, this therefore suggests an intriguing interplay between latent viral proteins in promoting survival and proliferative risks of KSHV-infected cells.

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